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Rapid and Sensitive Detection of Cell-Associated HIV-1 in Latently Infected Cell Lines and in Patient Cells Using Sodium-n-Butyrate Induction and RT-PCR

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To develop a rapid and sensitive means of detecting cell-associated human immunodeficiency virus (HIV), donor cells from HIV seropositive patients were treated with the potent viral activator sodium-n-butyrate (NaB) and subsequently assayed by both in situ RNA hybridization and a reverse transcriptase polymerase chain reaction (RT-PCR). The sensitivity of RT-PCR was estimated to be equivalent to 1×10^{-16} grams (0.1 fg) or approximately 64 copies of the input standard viral RNA per reaction. The present study takes advantage of the ability of NaB to introduce changes in chromatin structure of latently infected cells, leading to increased HIV gene expression. Human ACH-2 and U1 cell lines were used as representatives of T-lymphocytic and monocytoid cells harboring latent inducible proviruses. HIV gene expression was readily detected when these cells were treated with NaB. Viral *gag* RNA was detected by both in situ and RT-PCR assays. When peripheral blood mononuclear cells (PBMCs) from acquired immunodeficiency syndrome (AIDS) patients, who were all negative for *in situ* hybridization and serum/plasma p24 assays, were used for detection of viral gene expression, four categories with distinct patterns of induction were observed. The first set of patients showed HIV-positive PBMCs by RT-PCR without any added NaB, and suppression by added NaB or PHA. The second set of samples showed induction of viral RNA by NaB alone. The third set could be induced with PHA, but not NaB, and the fourth set required both NaB and PHA for induction of HIV gene expression. Our results suggest that direct treatment of the cells with HIV activators may be useful in increasing sensitivity of the RT-PCR intended to be used for detection of cell-associated viral RNAs. This approach may be used to confirm true status of the HIV infection when p24 results are negative or HIV RNAs in serum/plasma are below the threshold of detection. Moreover, this method may identify the presence of latent proviral genomes possibly reflecting the true rate of cell-associated viral load in vivo and without possible mutations brought about by long-term co-cultivation assays with cells from seronegative donors. ***J. Med. Virol. 52:179–189, 1997.***

KEY WORDS: lymphocytic/monocytoid cells; PBMC; diagnostics

INTRODUCTION

Progression of disease in human immunodeficiency virus (HIV) infected subjects is commonly measured indirectly using surrogate markers, such as CD4 cell count, β2-microglobulin or interferon levels, often supplemented with direct approaches measuring the viral load [Fang et al., 1995; Posnett et al., 1995]. Many different assays have been developed to determine the viral load qualitatively and quantitatively in serum/plasma and/or to monitor the effectiveness of antiviral drugs by measuring either viral RNA or antigen levels [Piatak et al., 1993; Ferre et al., 1993; Gupta et al., 1993; Urdea et al., 1993; Carton et al. 1994; Fiscus et al., 1995; Katzenstein et al., 1995; Dickover et al., 1996]. The sensitivity of a standard p24 assay, for detection of HIV in serum/plasma, is generally dependent on the time of infection or level of viremia [Kavlick et

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al., 1995; Kappes et al., 1995; Henrard et al., 1995]. Viral p24 antigen has also been detected in mitogen-stimulated PBMCs of seropositive individuals after culturing the cells for seven days [Kim et al., 1994]. Another marker of infection, namely cytotoxic T cell (CTL) reactivity, has also been examined in an effort to predict the course of disease [Yamamura et al., 1995; van der Burg et al., 1996]. The levels of cell-associated viral RNAs in donor cells, however, have not been extensively studied for either diagnostic or prognostic utility.

In certain asymptomatic individuals, HIV is found in latently infected cells as evident from the presence of HIV DNA, but not RNA in circulating PBMCs [Patterson et al., 1993]. Furthermore, in quiescent T cells from asymptomatic patients, HIV is present in an unintegrated proviral form with little or no significant level of viral gene expression [Bukrinsky et al., 1991]. Latent HIV infection is also seen in infiltrating lymphocytes within tumors [Embretson et al., 1993]. Levels of HIV transcripts in PBMCs directly correlate with intensity of viremia or stage of the disease [Lee et al., 1994; Melon Garcia et al., 1995]. There is also an inverse relationship between levels of the viral transcripts and CD4 counts, and cell-associated viral RNA is usually detected during clinical deterioration [Schnittman et al., 1991; Arens et al., 1993].

Evidence for the presence of latent HIV in different cell lines has been well established. HIV proviral genome in human lymphocytic ACH-2 and monocytic U1 cell lines can be maintained in latent state for a long period of time in culture [Folks et al., 1987, 1989]. These cells express extremely low levels of viral genes during latency and generally exhibit undetectable levels of full-length genomic or prespliced viral transcripts [Pomerantz et al., 1990; Sadaie and Hager, 1994]. Numerous agents ranging from chemicals such as phorbol myristate-13-acetic acid (PMA) to cytokines such as tumor necrosis factor-alpha (TNF) are known to activate HIV (Rosenberg and Fauci, 1989). Notably, sodium-n-butyrate (NaB), a short-chain fatty-acid, is a strong proliferation-arresting and differentiation-inducing compound which is capable of activating a number of human RNA and DNA viruses; e.g., HIV-1 [Golub et al., 1991; Laughlin et al., 1993; Sadaie and Hager, 1994], cytomegalovirus (CMV) [Tanaka et al., 1991], and Epstein Barr virus (EBV) [Luka et al., 1979; Contreras-Ssalazar et al., 1990]. NaB is thought to inhibit histone deacetylase activity, resulting in hyperacetylation of histone N-terminal tails [Riggs et al., 1977]. NaB is also postulated to affect topoisomerase II activity thereby changing a transcsriptionally silent chromatin to a transcriptionally competent chromatin [Sorenson et al., 1990].

To determine cell-associated viral burden we first treated latently infected ACH-2 and U1 cell lines and then donor cells, from HIV seropositive patients, with NaB, PHA, or a combination. We employed two different detection systems including an immune-based in situ hybridization and a reverse-transcriptase polymerase chain reaction (RT-PCR). We demonstrated that viral RNAs expressed in latently infected cell lines are readily detectable by in situ hybridization or RT-PCR only after treatment with activators such as NaB. However, the cell-associated viral RNA from patient PBMCs was detected only by RT-PCR, and not by digoxigenin-labeled in situ assay upon induction with NaB. Our results suggest that treatment of cells with NaB alone, or in combination with PHA within a short time period (24 hr post-induction), may increase the sensitivity of currently available diagnostic assays for the detection of cell-associated viral RNAs. This approach may also be used for detection of latent provirus in vivo, which often escapes detection by conventional hybridization or PCR methods. Finally, induction of cells latently infected with other retroviruses such as HTLV-I, SIV, BIV, or endogenous human retroviruses, may be feasible using the NaB-based induction assay.

MATERIALS AND METHODS

Reagents

Sodium-n-butyrate (NaB) was purchased from Sigma (St. Louis, MO). A stock solution of 1 M was prepared in distilled water and stored protected from light at 4°C for up to 3 months. Stock solutions of 100 U/μl TNF-α (R&D System, Minneapolis, MN), and 10 nM PMA (Sigma) were prepared and stored at −20°C up to 3 months. A stock solution of 2 mg/ml purified phytohaemagglutinin (PHA) from Murex Diagnostics Ltd. (Dartford, England), and were prepared in distilled water and stored at −70°C.

Cell Lines and Treatments

Human monocytoid U937 and U1 [Folks et al., 1987], and lymphocytoid CEM and ACH-2 [Folks et al., 1989] cells were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) and 10 μg/ml gentamycin. All cell culture reagents were purchased from GIBCO (Grand Island, NY). Cells were maintained routinely at a cell density of $0.5–1 \times 10^6$ cells/ml. They were counted and seeded at 0.5×10^6 cells in 2 ml complete culture medium per well in a 24-well plate (Costar, Cambridge, MA). Cells were treated with different activators in culture media and were incubated for 24 hr at 37°C. Cells were then collected and washed one time with Dulbecco's phosphate buffered saline (D-PBS) (Gibco). All liquid remaining after centrifugation was aspirated and the cell pellets were processed for RNA isolation and in situ hybridization.

Patients

Blood samples were collected randomly from HIV-1 seropositive individuals ($n = 9$) at stage III of the Centers for Disease Control criterion. These patients have been participating in a NIAID IRB-approved protocol (85-CC-95) conducted by Department of Transfusion Medicine, the US National Institutes of Health. Their CD4+ T lymphocyte counts ranged from 166–776 mm^3. All patients were confirmed positive for antibody against HIV-1 but negative for serum/plasma p24 an-

TABLE I. Patients' Immunological and Virological Results Compared to Cell-Associated HIV-1 Specific RNA in Uninduced and Induced PBMC

Category	Patient #	Stage	CD4 Count	Serum P24	Serum Ab	Current therapy	RT/PCR of gag region from samples			
							Untreated	+NaB	+PHA	NaB+PHA
	202	III	358	–	+	None	+	–	–	–
I.	185	III	566	–	+	None	+	–	–	–
	230	III	285	–	+	None	+	–	–	–
	124	III	776	–	+	ddI	–/+	++	++	+++
II.	162	III	351	–	+	None	–	++	ND	ND
	16	III	414	–	+	None	–	–	++	+
III.	61	III	373	–	+	None	–	–	++	+
	193	III	166	–	+	AZT, bactrim, antifungal	–	–	++	+
IV.	137	III	455	–	+	None	–	–	–	++

AZT (zidovudine); ddI (didanosine); ND (not done).

tigen. Only two patients had received prior therapies with anti-retroviral drugs (Table I).

Patient Cells and Treatments

Blood was collected from various HIV-1 infected individuals and the peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque and washed two times with D-PBS. The cells were seeded in four wells of a 24-well plate at concentrations of 0.5–0.75 × 10^6 cells each well in one ml of RPM1-1640 medium containing 10% fetal calf serum (FCS) and 10 μg/ml. One well was left untreated and the remaining wells were treated with either 5 mM NaB alone, 5 μg PHA alone or 5 mM NaB in combination with 5 μg PHA. The cells were incubated for 24 hr in an incubator at 37°C containing 5% CO_2. They were then collected and washed one time with D-PBS. All remaining liquid was aspirated and the cell pellets were stored at –70°C.

RNA Isolation and RT-PCR

Total RNA was extracted from the frozen cell samples by using TRIzol Reagent (Gibco) according to manufacturer's instructions. The precipitated RNA was resuspended in distilled water and the amount was quantitated by measuring the absorbance at 260 nm. RT-PCR was performed on the total RNA samples and the in vitro transcribed RNA standard with the use of the Thermostable rTth Reverse Transcriptase RNA PCR Kit as recommended by the manufacturer (Perkin-Elmer Cetus, Foster City, CA) and the DNA Thermal Cycler 480 (Perkin-Elmer Cetus). The primers utilized were HIV-1 *gag* primers [Udaykumar et al., 1994]; SR71 (5′-GCGGAGGCTAGAAGGAGAGAGA-3′, sense primer) and SR72 (5′-TCTTTCCCCCTGGCCTTAACCG-3′, antisense and cDNA primer) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers; (5′-TGGACACGGTTATAGACACG-3′, sense primer) and (5′-CAGTAGCGTAGATATACACG-3′, antisense and cDNA primer) [Frech et al., 1994]. The cDNA synthesis reaction was performed with the antisense primer, in the presence of $MnCl_2$ at 70°C for 15 min in a reaction volume of 20 μl. The DNA PCR amplification was performed with the addition of the sense primer, chelating buffer and $MgCl_2$ at 95°C for 120 sec for the first cycle, 95°C for 1 min, and 60°C for 1 min for 35 cycles, and then 60°C for 7 min for the final cycle in a reaction volume of 100 μl.

Detection of Amplified Fragments

The amplified samples were either electrophoresed on 2.5% agarose gels in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer or on 4–20% acrylamide gels (Novex, San Diego, CA) in TBE (45 mM Tris-borate, 1 mM EDTA) buffer and stained with ethidium bromide (0.5 μg/ml). Radioactive RT-PCR was performed first with cold nucleotides for reverse transcriptase reaction and subsequent DNA-PCR with 0.2 mM each of dATP, dGTP, dTTP, 0.02 mM dCTP, and 10 μCi of ^{32}P-dCTP (Dupont-NEN, Boston, MA). The final reaction volume was 100 μl for 35 cycle.

In Situ Hybridization

Cells (0.5 × 10^6) were treated with different activators and processed for in situ hybridization according to a standard procedure [Mitchell et al., 1992]. Briefly, cells were air-dried on Teflon-coated eight-well glass slides (~0.5 × 10^5/well), fixed with 40% formaldehyde and hybridized with specific probes. When necessary, slides were stored at –20°C under 70% ethanol. Digoxigenin-labeled HIV-1 genomic riboprobes were obtained using a pool of run-off transcripts derived from overlapping proviral fragments which were subcloned into SP65 vectors (Lofstrand Labs Ltd., Gaithersburg, MD). All the probes and reagents were validated in quality control runs before the actual assay was performed. Anti-digoxigenin alkaline phosphatase antibody and the substrate color development was carried out for 2 hr, and according to manufacturers's suggestions (Boehringer Mannheim, Indianapolis, IN). The slides were assayed in duplicate, receiving separate amounts of anti-sense and sense probes predetermined to be optimal for hybridization with minimal background noise.

Construction of RNA Standard

Two synthetic oligonucleotides, each 76 bases in length, representing the HIV-1 *gag* were synthesized. They span the complementary SR71 and SR72 primer regions and contain a 30 bp deletion. The sense strand contained a *Kpn1* restriction site at its 5′ end and the antisense strand contained a *Sac1* restriction site at its

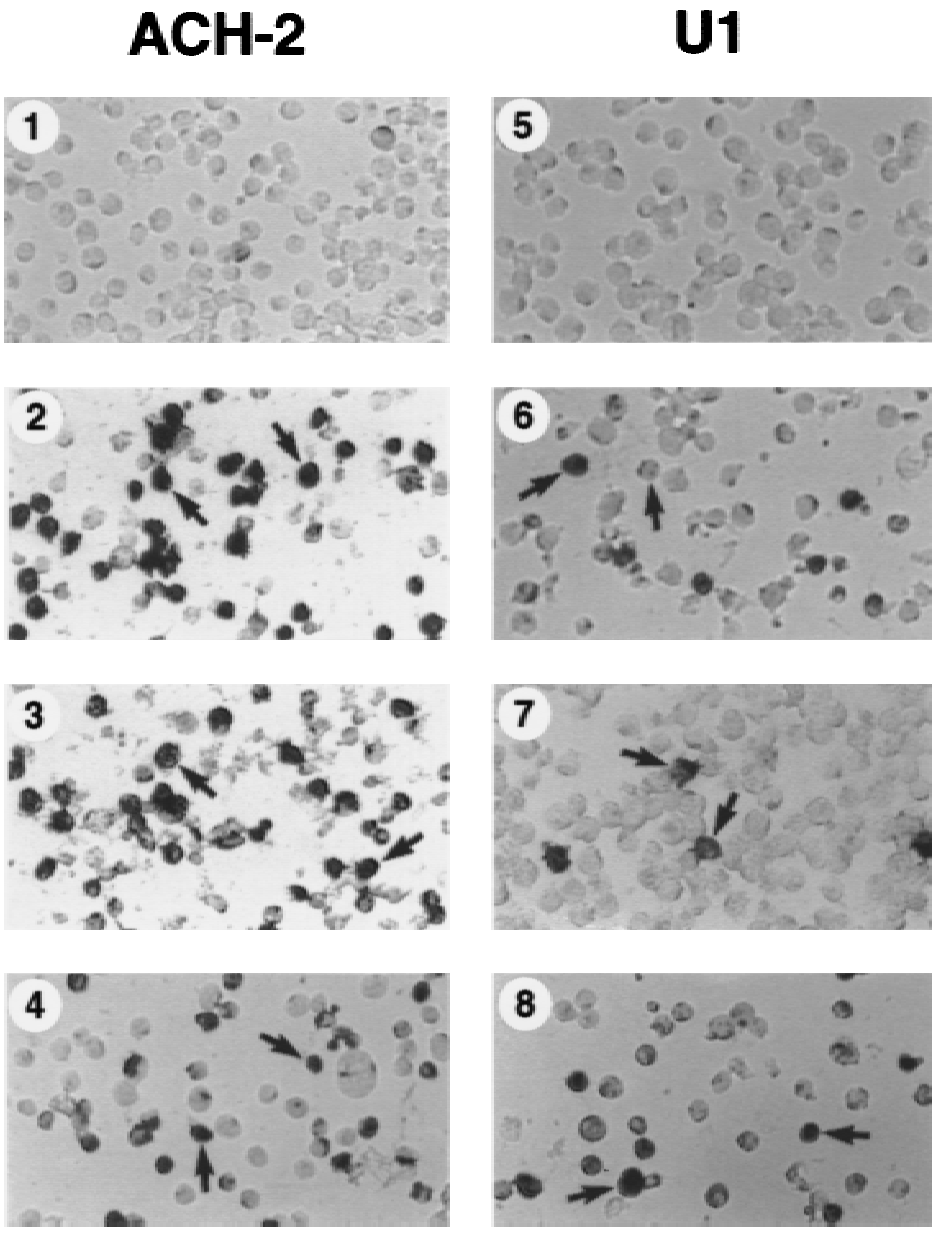

Fig. 1. Detection of HIV-1 RNA transcripts in latently infected ACH-2 and U1 cells. **Panels 1–4**: (ACH-2) and **5–8** (U1) represent untreated (1 and 5), and treated with 5 mM NaB (2 and 6), 500 units/ml TNF (3 and 7), 100 μM PMA (4 and 8), respectively. The arrows indicate examples of stained cells which contain HIV-1 transcripts that have been hybridized to the specific digoxigenin labeled antisense probe and visualized by in situ hybridization as described in Materials and Methods. In panel 6, the arrow on the right represents one example of a negative cell. In each case a total of 200 cells were counted and scored for positive hybridization.

3′ end. One μg of each of the two oligonucleotides were annealed together in a buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 50 mM NaCl by boiling for 5 min and then cooled slowly to room temperature for 45 min. The annealed oligonucleotides were then cloned into pBluescript SK M13 (+) phagemid (Stratagene, La Jolla, CA) using standard subcloning techniques. In vitro transcription was performed using T3 RNA polymerase (Promega, Madison, WI), and run-off transcripts of ~81 bases in size were gel purified using a 4% PAGE, quantitated, and stored in aliquots at −70°C.

Calculation of RNA Copy Number

Pictures of ethidium bromide-stained gels containing RT-PCR products of known amount of standard RNA were scanned using the ImageQuant scanner (Molecular Dynamics, Personal Densitometer). Comparisons of scanned gels containing ACH-2, U1, and patient samples were calculated based on this comparison. We estimated that 1 ng of 100 base RNA represents 1.8×10^7 molecules of *gag* product from infected cells. Control amplified *gag* product, derived from run-off transcripts (~81 bases), was used as standard control at

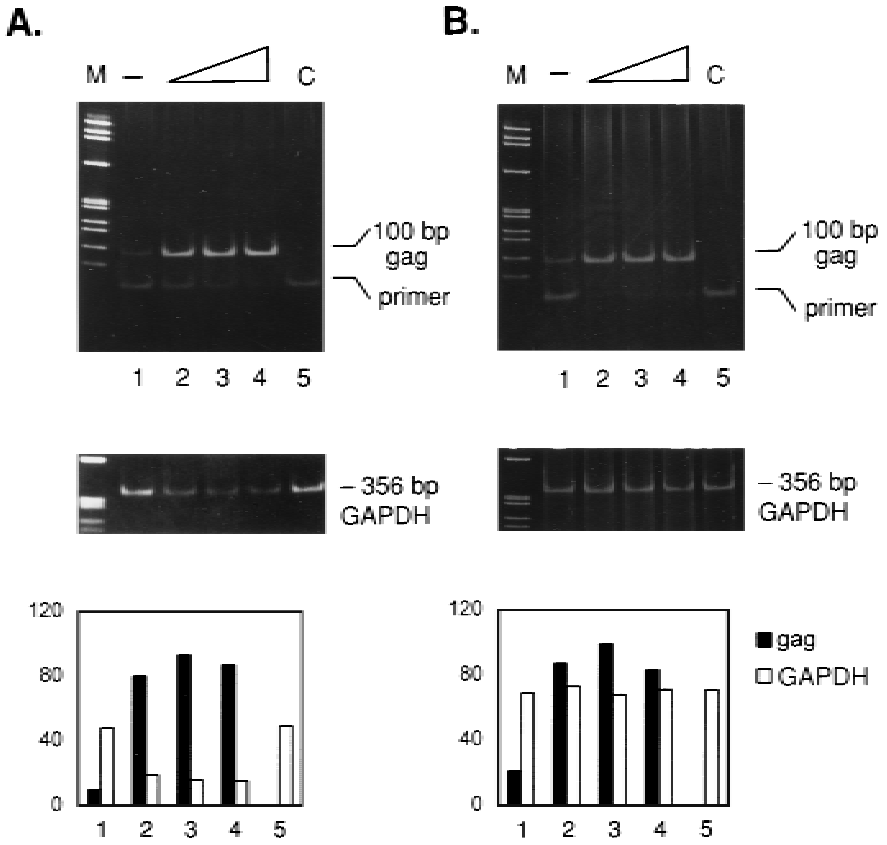

Fig. 2. Dose-response effect of NaB on the activation of HIV-1 transcripts in ACH-2 and U1 cells. RT-PCR products from total cellular RNA from ACH-2 **(A)** or U1 **(B)** cells were amplified with SR71/SR72 *gag* primers (upper panels) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (lower panels). Samples were analyzed by gel electrophoresis in 4–20% acrylamide gels and stained with ethidium-bromide. Lane(s): (M) 0.5 μg of PhiX174 RF DNA/*Hae* III fragments, (1-4)100 bp *gag* product or the 356 bp GAPDH product corresponding to NaB concentrations of 0, 1, 5, and 10 mM, respectively, (5) RNA from untreated, uninfected parental CEM (left panel) or U937 (right panel) cells (negative controls). Bottom bar graphs represent intensity of bands of *gag* and GAPDH using quantitative densitometry.

0.81 ng, representing 1.8×10^7 molecules, in combination with total RNA from uninfected U937 cells.

RESULTS

Detection of HIV-1 RNA by In Situ in Latently Infected Cell Lines

The effect of NaB and some other activators (as positive controls) such as TNF and PMA was examined first in the latently infected lymphocytic ACH-2 and monocytoid U1 cells by in situ hybridization (Fig. 1). Both TNF and PMA have been suggested as HIV inducers. NaB is a potent activator of the HIV-1 gene expression in ACH-2 cells; accounting for more than 90% of the positive cells 24 hr after the treatment with this compound. The number of strong positive ACH-2 cells expressing the viral transcripts were ~50% higher in the NaB-treated lymphocytic cells than the same cells treated with TNF (Fig. 1, panels 2 and 3). Moreover, the integrity or morphology of the NaB-treated cells was better preserved than with TNF. Interestingly, it was found that there are marked differences between the lymphocytic ACH-2 and monocytoid U1 cells in terms of their response to different activators. For instance, the PMA-treated ACH-2 cells showed fewer positive cells (panel 4) than ACH-2 cells treated with NaB or TNF (panels 2, 3). All three compounds (NaB, TNF, PMA) tested were relatively more effective in the induction of HIV-1 in ACH-2 cells than U1. The latter demonstrated fewer positive cells, presumably due to the presence of some uninducible cells in this cloned cell line.

Enhanced Sensitivity of RT-PCR for Detection of HIV-1 RNA in Latently Infected Cell Lines

To determine the effect of NaB on detectability of HIV transcripts by RT-PCR assay, we first performed dose response analysis of this compound on lymphocytic ACH-2 and moncytoid U1 cells. Total RNA from these cells were amplified by RT-PCR using SR71/SR72 *gag* primers. The RNA samples from NaB-treated (1, 5, and 10 mM) cells clearly demonstrated substantial increase; about nine-fold (~90%) for ACH-2, and five-fold for U1 in the HIV-1 *gag* (100 bp) RT-PCR product as indicated in the representative Figure 2. Densitometric scanning of the RT-PCR results is shown graphically in Figure 2 bottom panel. The amplified product increases were consistently higher for ACH-2 cells than U1, and as confirmed by end-point sensitivity described below. These appear to correlate with the HIV-1 RNA expression seen by in situ hybridization as indicated in Figure

1, and suggest that the addition of NaB to these cells upregulates viral gene expression and thus increases the sensitivity of RT-PCR.

RNA from both cell types were also amplified using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers to show input RNA equivalence in the RT-PCR. In ACH-2 cells, the increase in HIV-1 *gag* product was inversely proportional to the level of endogenous GAPDH. The reduction in the representative GAPDH product was perhaps due to downregulation of the endogenous corresponding gene or due to reduction in its stability in presence of NaB. Consistent with RT-PCR results, we have observed a similar reduction in steady-state levels of GAPDH in Northern blots from these cells [Moulton and Sadaie, unpublished data].

End-Point Sensitivity of RT-PCR on RNA From Latently Infected Cells

Since NaB could increase the amount of HIV-1 transcripts in latently infected cell lines, it was of interest to see what the limit of detection of the RT-PCR amplified samples would be by simple ethidium-bromide staining. For this experiment we used ACH-2 and U1 cells that were treated with 5 mM NaB. Figure 3 (A and B) are representative of RT-PCR products from RNA from ACH-2 and U1 cells, respectively, amplified with SR71/SR72 *gag* primers and analyzed on an agarose gel stained with ethidium-bromide. After scanning the gel and comparing to the standard RNA, we found that the HIV-1 specific *gag* transcripts of NaB-treated ACH-2 and U1 cells could be detected with as little 0.1 pg and 1 pg of total cellular input RNA, respectively.

Sensitivity of RT-PCR on *gag* Standard RNA

HIV-1 *gag* specific standard RNA (see Methods) was used to quantitate the copy number of input HIV-1 template in our experiments. The in vitro run-off RNA products from this clone, when amplified by RT-PCR with SR71/SR72 primers, produces a 70 bp product. Figure 4 shows the result of such an experiment, where constant amounts of uninfected U937 total RNA were mixed with varying amounts of standard RNA in RT-PCR reactions. A densitometric scan of the 70 bp product is shown in the same figure where as little as 0.1 fg of input RNA can be detected when mixed with total cellular RNA. Therefore, we concluded from these experiments that the sensitivity of RT-PCR using total cellular RNA from HIV-infected cells is around 0.1 fg.

Ethidium Bromide Staining and Radioactive Method in Detection of End-Point Sensitivity

We then asked if a similar pattern of sensitivity could be observed using standard ethidium bromide staining assay. Figure 5 shows a titration of the standard RNA amplified by RT-PCR, detected by gel electrophoresis and ethidium bromide staining. The bottom panel shows the same titration with radioactive RT-PCR. We could detect as little as 1×10^{-16} grams (0.1 fg) or 64 copies of input standard RNA both by ethidium-bromide staining and radioactive RT-PCR.

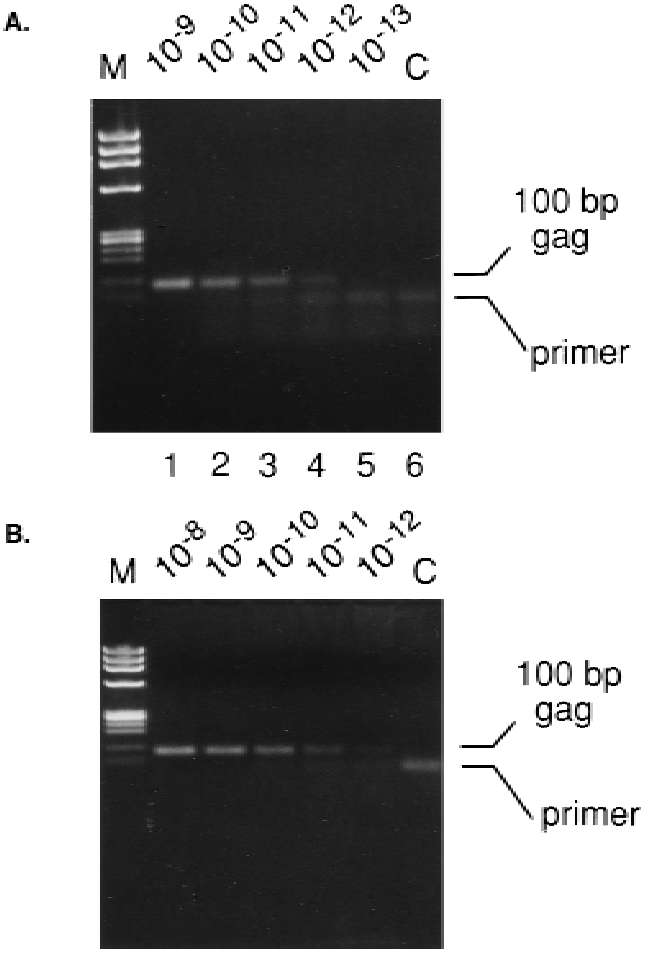

Fig. 3. **A**: Sensitivity of RT-PCR products of HIV-1 *gag* from cell lines treated with NaB. (A) represents serial dilution of total cellular RNA from ACH-2 cells. *Gag* product of 100 bp can be observed from samples diluted up to 0.1 pg of total RNA (lane 5). **B**: U1 cells treated with 5 mM NaB, and serially diluted for *gag* RT-PCR. A *gag* product can be observed up to 1 pg of total cellular RNA. M represents PhiX174 RF DNA/*Hae* III fragments. C, blank (no RNA).

According to conditions used here, where only one radiolabled nucleotide was used, we concluded that ethidium bromide detection was as sensitive as the radioactive method in detecting *gag* specific RNAs.

RT-PCR of Patient PBMC Samples

Since the previous results showed that NaB increases sensitivity of RT-PCR in detecting HIV-1 RNA from latently infected cell lines, it was of interest to see if it would have the same effect on PBMCs collected from HIV-1-infected individuals. Therefore, the patient PBMCs were collected and treated with NaB alone, or in combination with PHA, a mitogenic stimulator of T-cells. Nine HIV-1 infected subjects whom were all confirmed antibody positive, but were negative for serum/plasma p24 Gag antigen, were screened in this study. Briefly, 0.5×10^6 PBMC patient cells were obtained using Ficoll-Paque, washed with PBS and cultured for 24 hr in the presence or absence of inducers.

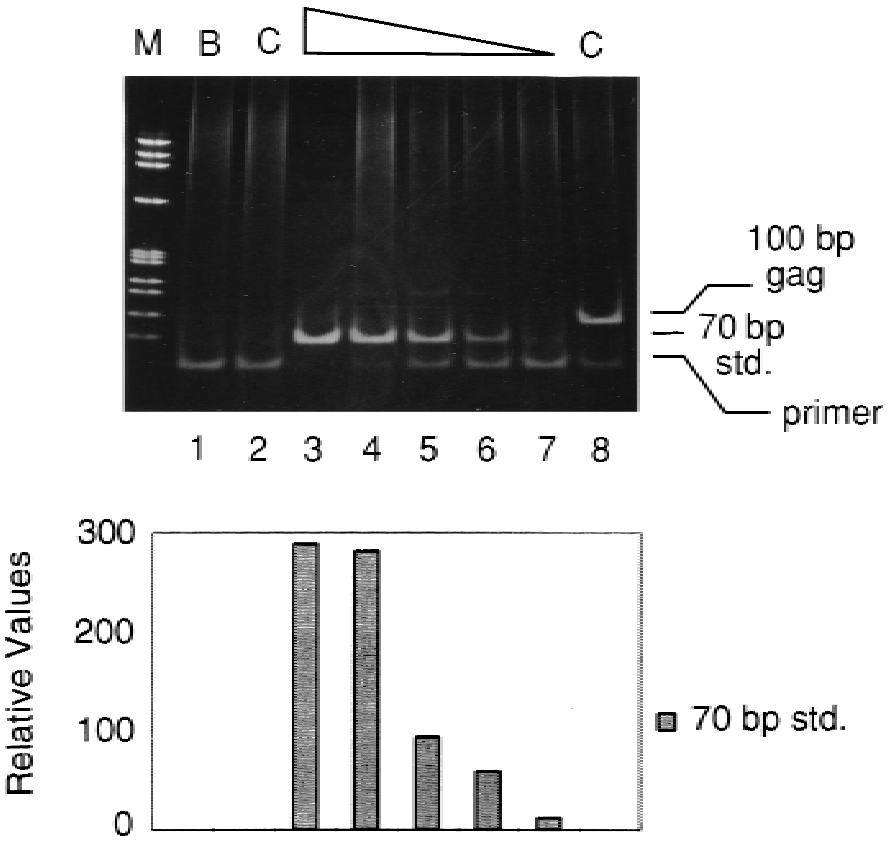

Fig. 4. Analysis of HIV-1 specific RNA standard (70 bp) sensitivity in RT-PCR using *gag* primers. **Lane 1, B**: RT-PCR with no exogenously added RNA. **Lane 2, C**: RT-PCR from uninfected U937 cells (1 ng of total cellular RNA). **Lanes 3–7**: RT-PCR from serially diluted standard RNA (1×10^{-13} to 1×10^{-17} g) mixed with 1 ng of total cellular RNA from U937 cells. **Lane 8**: 1 ng of total cellular RNA from U1 cells treated with 5 mM NaB. Bottom graph corresponds to lanes 1–7 of scanned gels for quantitation of 70 bp *gag* standard product. Lane M, phiX174 RFDNA/*Hae* III fragments.

Cells were then collected and processed to obtain total cellular RNA for RT-PCR. Results of a representative experiment are shown in Figure 6, where *gag* RNA can be readily detected once cells were stimulated by NaB (top panel, lane 2). RNA samples of this particular patient also showed an identical RT-PCR product derived from PHA-treated lymphocytes (lane 3). Moreover, cells concurrently treated with NaB+PHA showed consistently a stronger signal (lane 4). The integrity of RNAs were also tested using GAPDH primers. Figure 6, bottom panel, shows a 356 bp GAPDH product of the corresponding patient RNAs. A composite of all of the nine patient samples treated with NaB and/or PHA is shown in Table I. We have concluded from this study that all of nine patient's lymphocytes had expressed HIV genes once cultured for 24 hr. These results indicate that there were four categories of AIDS patients based on induction of latent virus and detected by RT-PCR. Category 1 showed *gag* RNA without the addition of inducers. In fact, various inducers such as NaB and PHA down-regulated the HIV RNA levels in these patients. Category 2 were negative in the absence of inducers, but once stimulated with NaB, they produced virus detectable by RT-PCR. The third category was also negative in the absence of inducers, but could only be stimulated with PHA and not NaB. Finally, the fourth category would show no virus RNA production unless treated with both NaB and PHA. Therefore, all patients (who were initially negative for serum/plasma p24 antigen) showed virus gene expression once cultured in vitro for 24 hr or induced with activating agents such as NaB and/or PHA. Production of extracellular virus in the cultures was not confirmed.

DISCUSSION

Although a variety of useful diagnostic assays with distinctly different performance characteristics are now available to monitor HIV infection, each appears to have certain advantages and limitations. Antibody screening assays do not measure virus in clinical specimens, but are useful to detect infection. However, if HIV vaccines are widely used in the future, serological assays may not discriminate between the natural host response to vaccine and potential post-vaccination infection. This could occur especially with certain replicative vaccines or DNA vaccinations expressing multiple viral proteins. Surrogate markers such as CD4 T cell count, interferon, or β2-microglobulin levels measure disease activity but only indirectly. Thus, the need for a direct measurement of the virus in clinical speci-

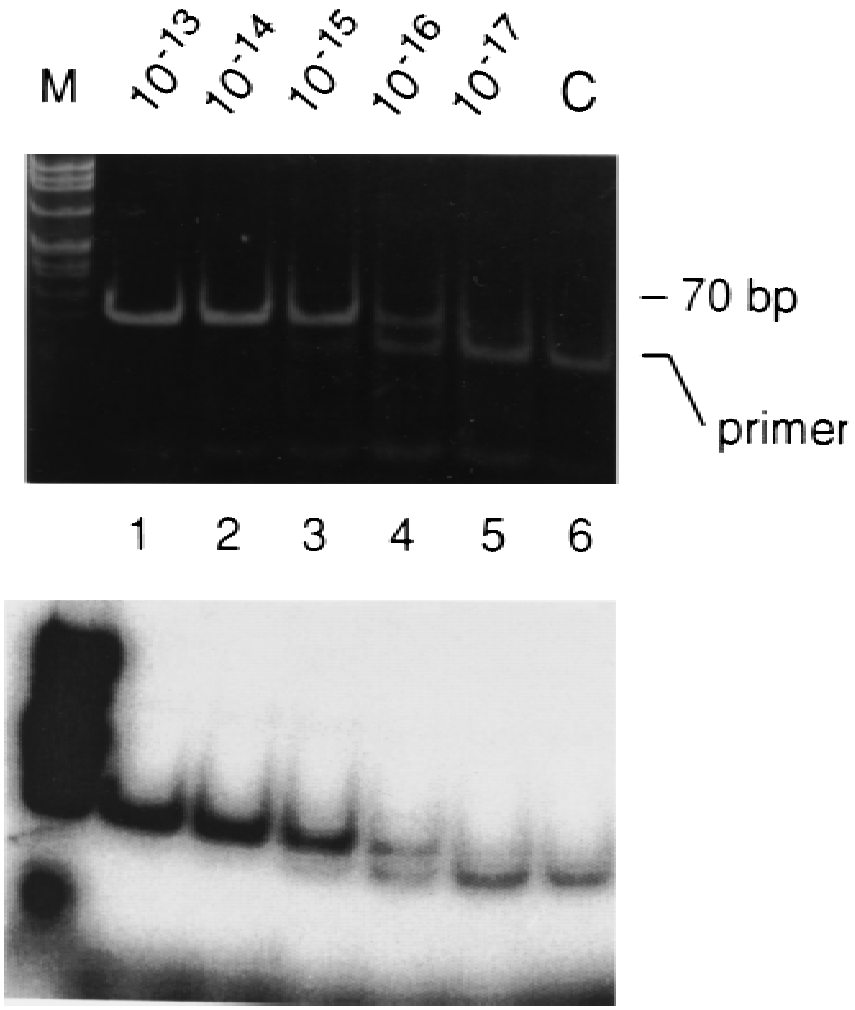

Fig. 5. Detection of end point sensitivity of standard RNA using ethidium bromide and radioactive methods. **Top panel**: Ethidium bromide staining of gels containing 70 bp *gag* standard product. **Lane C**: 1 ng of U937 total cellular RNA used for RT-PCR. **Bottom panel**: Identical to top panel, but a radioactive nucleotide was added to the RT-PCR reaction. Products can be observed in both top and bottom panels in lanes containing up to 0.1 fg or 64 copies of RNA.

mens is warranted, both for diagnostic and prognostic purposes.

The emphasis of the present work is placed on the development of a rapid and sensitive RNA assay monitoring viral gene expression in a shortest possible time. Since this assay is looking at cell-associated RNA, it has an advantage over PCR and p24 antigen. Although we tested only a single sample from each of the patients, this work has shown that this method would be a quick and facile way to monitor virus in patients participating in longitudinal studies, especially in evaluating efficacy of treatment regimens. We opted to place emphasis on the importance of the monitoring circulating cell-associated virus from a diagnostic point of view. RNA-PCR is useful in monitoring: a) state of viral replication, b) effectiveness of anti-viral therapy, c) nature of infection where proviral DNA copy number is extremely low. A simplified diagram comparing various approaches to screen for the presence of HIV in plasma or PBMCs is depicted in Figure 7. Briefly, PBMCs obtained from the buffy-coat can be cultured for increased viral transcription, in the presence of inducers NaB and/or PHA in a 12 to 24 hr period. The cell-associated RNA can then be isolated and used for an RNA-PCR assay. In parallel studies, we also obtained plasma RNA, from all HIV-1 stage III patients, and observed no amplification of *gag* RNA (data not shown). This is an important point since conventional RNA-PCR assays often fail to detect viral particles in serum/plasma of asymptomatic stage III patients. It is interesting to note that eight out of nine patient PBMCs were positive for virus release, once co-cultured with normal PBMCs in a 5 week period (data not shown). However, this rate of detection is effective only by means of co-cultivation which presumably induces the patient PBMCs. Although this method can directly measure the virus, it appears limited for several reasons: 1) any cultures maintained for more than one day can increase the chance of contamination with adventitious agents; 2) long-term cell cultures measure

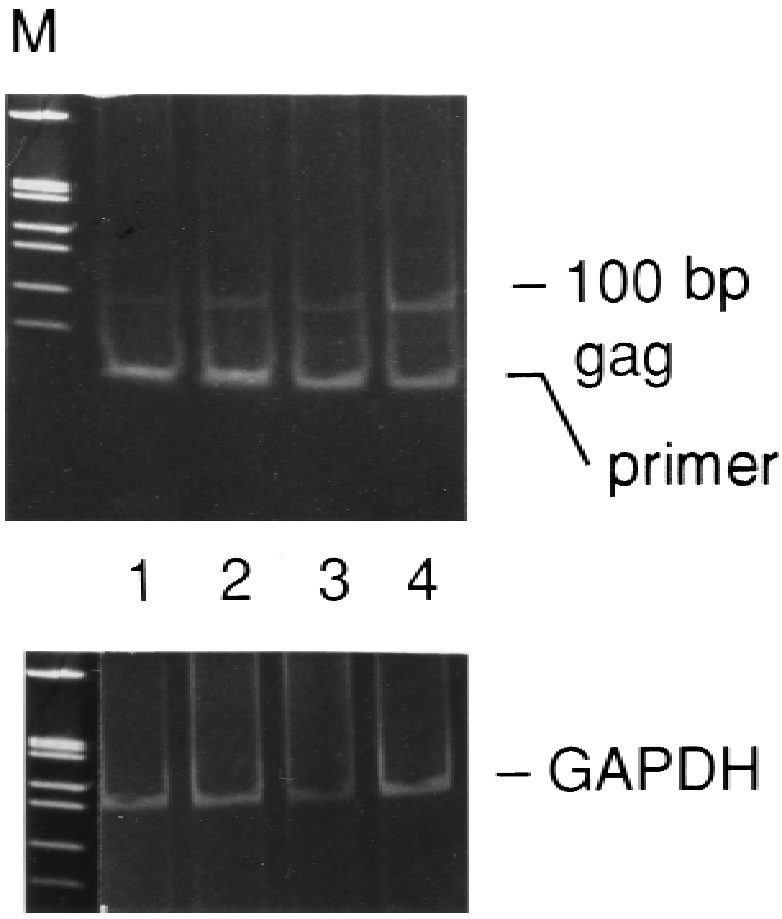

Fig. 6. A representative of RT-PCR from patient PBMCs. Cells from patient #124 (see Table 1 for details) were used for RT-PCR reactions containing either *gag* or GAPDH primers. **Lane 1**: Total cellular RNA from untreated cells. **Lanes 2–4**: RT-PCR from PBMCs treated with NaB (5 mM), PHA (5 μg/ml), NaB (5 mM) + PHA (5 μg/ml), respectively. Total cellular RNA (5 ng) was amplified using either *gag* primers (**top panel**) or GAPDH primers (**bottom panel**).

accumulated progeny viruses; presumably resulting from multiple rounds of virus replication which can bias growth of a particular virus strain, with respect to different HIV subtypes, quasi-species, or clades [Myers et al., 1994]; 3) coculture assays are usually costly for routine rapid screening.

It should be mentioned that in non-human primates, i.e., chimpanzees, induction of HIV in PBMCs has been reported by culturing the cells with inducers such as PHA and PMA [Saksela et al., 1993]. However, the same technique applied to human non-progressor patient PBMCs has not been successful [Garbuglia et al., 1995]. Therefore, it was of considerable interest to us to be able to detect viral genome from a cell population with a low infection rate, i.e., PBMCs as opposed to clonal cell lines such as lymphocytic ACH-2 cells where 100% of cells are infected with HIV genome.

Our rapid method is completed in 1 day. Based on viral genetics, the observed signal in RT-PCR represents HIV gene expression from probably only one round of viral replication. Further, it utilizes a minimal amount of patient cells, i.e., in the amount of not more than 0.5×10^6 donor cells as opposed to 10^7 cells previously utilized in a 7-day direct culture and subsequent p24 assay [Kim et al., 1994]. Also, our approach combines the inherent sensitivity of the RT-PCR with already amplified viral gene expression because of the treatment with various stimulators, NaB, and/or PHA. This leads to an additional several-fold amplification of HIV specific RNA signal, thus achieving a rapid and superior sensitivity level of detection. The latter also lacks a potential false positive reactivities which could occur with antibodies utilized in p24 assay systems. Such antibodies often react non-specifically with secreted nonviral cellular products in culture supernatants, especially when p24 assays are performed with lower specimen dilutions.

Interestingly, it has been demonstrated that when HIV-infected individuals were vaccinated with clinically approved non-HIV vaccines, HIV viral load in the vaccinated subjects were transiently increased [O'Brien et al., 1995]. This report further supports the contention that a significant portion of the viral load in vivo is present in "latent" form with potential ability to replicate upon stimulation. Therefore, induction of a pre-existing silent proviral genome by means of stimulus-induced upregulation, could become a useful diagnostic method as compared to other assays such as

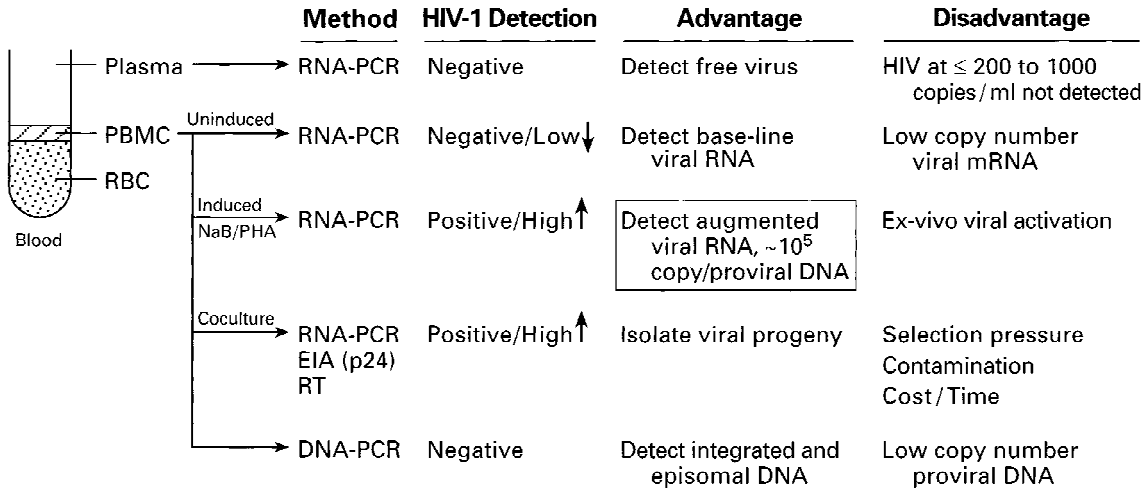

Fig. 7. Schematic representation of various commonly used HIV-1 detection procedures as compared to induced cell-associated virus. Fractionated whole blood is processed for RNA-PCR using either uninduced or induced PBMCs upon NaB and/or PHA treatment, or cocultivation with uninfected donor cells. It is estimated that one HIV-1 proviral DNA results in the expression of roughly 10^5 copies of viral RNA [Dimitrov and Martin, 1995].

DNA-PCR based techniques. Furthermore, contrary to plasma viremia or DNA-PCR, cell-associated viral RNA, i.e., RT-PCR, should provide direct assessment of the expression or regulation of HIV within patient cells and this is likely to correlate well with the state of infection.

Therefore, results presented here show that NaB, PHA, or a combination of NaB+PHA are capable of increasing the sensitivity of detection of HIV in specimens from stage III patients (Table I). This approach could be useful in obtaining viral sequences from longitudinal studies of asymptomatic carriers. The present study also confirms independent reports that HIV-1 genes are detected upon stimulation by PHA and PMA [Saksela et al., 1993; Tetali et al., 1993]. Both reports have observed increased sensitivity of detection by PCR when using PHA and PMA, correlating with induction of transcription factors, like NF-kB. The results from the current study could imply a similar path of induction or in part could directly involve changes in chromatin structure/function.

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